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Early Selection in Gag by Protective HLA Alleles Contributes to Reduced HIV-1 Replication Capacity That May Be Largely Compensated for in Chronic Infection▽†

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Mutations that allow escape from CD8 T-cell responses are common in HIV-1 and may attenuate pathogenesis by reducing viral fitness. While this has been demonstrated for individual cases, a systematic investigation of the consequence of HLA class I-mediated selection on HIV-1 *in vitro* replication capacity (RC) has not been undertaken. We examined this question by generating recombinant viruses expressing plasma HIV-1 RNA-derived Gag-Protease sequences from 66 acute/early and 803 chronic untreated subtype B-infected individuals in an NL4-3 background and measuring their RCs using a green fluorescent protein (GFP) reporter CD4 T-cell assay. In acute/early infection, viruses derived from individuals expressing the protective alleles HLA-B*57, -B*5801, and/or -B*13 displayed significantly lower RCs than did viruses from individuals lacking these alleles ($P < 0.05$). Furthermore, acute/early RC inversely correlated with the presence of HLA-B-associated Gag polymorphisms ($R = -0.27$; $P = 0.03$), suggesting a cumulative effect of primary escape mutations on fitness during the first months of infection. At the chronic stage of infection, no strong correlations were observed between RC and protective HLA-B alleles or with the presence of HLA-B-associated polymorphisms restricted by protective alleles despite increased statistical power to detect these associations. However, RC correlated positively with the presence of known compensatory mutations in chronic viruses from B*57-expressing individuals harboring the Gag T242N mutation ($n = 50$; $R = 0.36$; $P = 0.01$), suggesting that the rescue of fitness defects occurred through mutations at secondary sites. Additional mutations in Gag that may modulate the impact of the T242N mutation on RC were identified. A modest inverse correlation was observed between RC and CD4 cell count in chronic infection ($R = -0.17$; $P < 0.0001$), suggesting that Gag-Protease RC could increase over the disease course. Notably, this association was stronger for individuals who expressed B*57, B*58, or B*13 ($R = -0.27$; $P = 0.004$). Taken together, these data indicate that certain protective HLA alleles contribute to early defects in HIV-1 fitness through the selection of detrimental mutations in Gag; however, these effects wane as compensatory mutations accumulate in chronic infection. The long-term control of HIV-1 in some persons who express protective alleles suggests that early fitness hits may provide lasting benefits.

The host immune response elicited by $CD8^+$ cytotoxic T lymphocytes (CTLs) is a major contributor to viral control following human immunodeficiency virus type 1 (HIV-1) infection (6, 39), but antiviral pressure exerted by CTLs is diminished by the selection of escape mutations in targeted regions throughout the viral proteome (7, 18, 29, 35, 41, 45, 57). A comprehensive identification of HLA-associated viral polymorphisms has recently been achieved through population-based analyses of HIV-1 sequences and HLA class I types from different cohorts worldwide (3, 8, 13–15, 34, 43, 50, 56, 63). However, despite improved characterization of the sites and pathways of immune escape, effective ways to incorporate these findings into immunogen design remain an area of debate. A better understanding of the impact of escape mutations on viral fitness may provide novel directions for HIV-1 vaccines that are designed to attenuate pathogenesis.

The development of innovative vaccine strategies that can

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overcome the extreme diversity of HIV is a key priority (4). One proposed approach is to target the most conserved T-cell epitopes, which presumably cannot escape from CTL pressure easily due to structural or functional constraints on the viral protein (55). Complementary approaches include the design of polyvalent and/or mosaic immunogens that incorporate commonly observed viral diversity (4, 38) or the specific targeting of vulnerable regions of the viral proteome that do escape but only at a substantial cost to viral replication capacity (RC) (1, 40). A chief target of such vaccine approaches is the major HIV-1 structural protein Gag, which is known to be highly immunogenic and to elicit CTL responses that correlate with the natural control of infection (22, 36, 66). Indeed, several lines of evidence support a relationship between the selection of CTL escape mutations and reduced HIV-1 fitness. These include the reversion of escape mutations following transmission to an HLA-mismatched recipient who cannot target the epitope (19, 24, 41) as well as reduced plasma viral load (pVL) set point following the transmission of certain escape variants from donors who expressed protective HLA alleles (17, 27). Notably, these *in vivo* observations have been made most often for variations within Gag that are attributed to CTL responses restricted by the protective alleles HLA-B*57 and -B*5801 (17, 19, 27, 41). Most recently, reduced *in vitro* RCs of clinical isolates and/or engineered strains encoding single or multiple escape mutations in Gag selected in the context of certain protective HLA alleles, including B*57, B*5801, B*27, and B*13, have been demonstrated (9, 10, 42, 53, 59, 62). Despite these efforts, the goal of a T-cell vaccine that targets highly conserved and attenuation-inducing sites is hampered by a lack of knowledge concerning the contribution of most escape mutations to HIV-1 fitness as well as a poor understanding of the relative influence of HLA on the viral RC at different stages of infection.

The mutability of HIV-1 permits the generation of progeny viruses encoding compensatory mutations that restore normal protein function and/or viral fitness. Detailed studies have demonstrated that the *in vitro* RC of escape variants in human and primate immunodeficiency viruses can be enhanced by the addition of secondary mutations outside the targeted epitope (10, 20, 52, 59, 65). Thus, vaccine strategies aimed at attenuating HIV-1 must also consider, among other factors, the frequency, time course, and extent to which compensation might overcome attenuation mediated by CTL-induced escape. Despite its anticipated utility for HIV-1 vaccine design, systematic studies to examine the consequences of naturally occurring CTL escape and compensatory mutations on viral RC have not been undertaken.

We have described previously an *in vitro* recombinant viral assay to examine the impact of Gag-Protease mutations on HIV-1 RC (47, 49). Gag and protease have been included in each virus to minimize the impact of sequence polymorphisms at Gag cleavage sites, which coevolve with changes in protease (5, 37). Using this approach, we have demonstrated that viruses derived from HIV-1 controllers replicated significantly less well than those derived from noncontrollers and that these differences were detectable at both the acute/early (49) and chronic (47) stages. Escape mutations in Gag associated with the protective HLA-B*57 allele, as well as putative compensatory mutations outside known CTL epitopes, contributed to this difference in RC (47). However, substantial variability was observed for viruses from controllers and noncontrollers, indicating that additional factors were likely to be involved. Benefits of this assay include its relatively high-throughput capacity as well as the fact that clinically derived HIV-1 sequences are used in their entirety. Thus, it is possible to examine a large number of "real-world" Gag-Protease sequences, to define an RC value for each one, and to identify sequences within the population of recombinant strains that are responsible for RC differences.

Here, we use this recombinant virus approach to examine the contribution of HLA-associated immune pressure on Gag-Protease RC during acute/early ($n = 66$) and chronic ($n = 803$) infections in the context of naturally occurring HIV-1 subtype B isolates from untreated individuals. In a recent report (64), we employed this system to examine the Gag-Protease RC in a similar cohort of chronic HIV-1 subtype C-infected individuals. The results of these studies provide important insights into the roles of immune pressure and fitness constraints on HIV-1 evolution that may contribute to the rational design of an effective vaccine.

MATERIALS AND METHODS

Patients and samples. The acute/early cohort was comprised of 66 antiretroviral-naïve individuals (median time postinfection of 61 days [interquartile range {IQR}, 37 to 74 days]; median pVL of 5.5 $\log_{10}$ RNA copies/ml [IQR, 4.0 to 5.9 $\log_{10}$ RNA copies/ml]; median CD4 count of 483 cells/mm^3 [IQR, 401 to 654 cells/mm^3]) from Acute Infection and Early Disease Research Program (AIEDRP) network sites in the United States and Australia as well as a private medical clinic in Germany (11). Thirty-nine patients were identified during acute infection as defined by documented positive HIV RNA (>5,000 copies/ml) or detectable serum p24 antigen and either a negative HIV-1 enzyme immunoassay (EIA) result or a positive EIA result but a negative or indeterminate Western blot result. The time frame for acute infection as defined here ranges up to 6 weeks following infection, and sample distribution is comparable to Fiebig stages 1 to 2 ($n = 11$) and 3, 4, and 5 ($n = 28$) (23). The remaining 27 individuals were identified during early HIV infection, as defined by a negative EIA result during the previous 6 months or a positive EIA result but a negative detuned HIV-1 EIA result (Vironostika-LS EIA; bioMérieux, Raleigh, NC) (33) at enrollment. These samples are comparable to Fiebig stage 6. The date of HIV infection was estimated using clinical history (where available), by subtracting 4 weeks from the baseline in cases of a negative EIA result, by subtracting 6 weeks from the baseline in cases of a positive EIA result, by calculating the midpoint between the last negative and the first positive EIA result, or by subtracting 4 months from the date of a negative detuned EIA result (11).

The chronic cohort was comprised of 803 individuals (median pVL of 5.1 $\log_{10}$ RNA copies/ml [IQR, 4.7 to 5.5 $\log_{10}$ RNA copies/ml] and median CD4 cell count of 273 cells/mm^3 [IQR, 130 to 420 cells/mm^3]). Of these individuals, 762 (94.9%) represented a baseline (antiretroviral-naïve) cross-section of the British Columbia HOMER cohort (12–14). The remaining 41 (5.1%) individuals represented chronically infected individuals recruited from Massachusetts General Hospital who were untreated at the time of sample collection (47). Although time since infection was unknown for these individuals, the relatively low median CD4 cell count indicates that this cohort was relatively progressed and thus may not be entirely representative of earlier stages of chronic infection. HLA class I typing was performed by using sequence-based methods. Ethical approval was obtained through the relevant institutional review boards.

Generation of recombinant Gag-Protease viruses. Recombinant viruses were generated on an NL4-3 background as described previously (47, 49). NL4-3 was chosen because this strain is commonly used for *in vitro* mutagenesis studies of HIV-1 replication, and its Gag sequence displays greater similarity to consensus subtype B than to other available molecular clones (13 amino acid differences from consensus subtype B, 2004). Briefly, the Gag-Protease region was amplified by reverse transcription (RT)-PCR from plasma HIV-1 RNA using sequence-specific primers. Second-round PCR was performed by using PAGE-purified recombination primers designed to match the NL4-3 sequence directly upstream of Gag (forward primer GACTC GGCTT GCTGA AGCGC GCACG GCAAG

TABLE 1. Acute/early cohort information

Parameter	Value for acute/early cohort			*P* value[b]
	Total	Protective HLA allele[a]	No protective HLA allele	
No. of individuals	66	20	46	
Median $\log_{10}$ plasma viral load (IQR[c])	5.47 (4.05–5.88)	4.44 (3.76–5.61)	5.59 (4.97–5.97)	0.02
Median CD4 cell count (IQR)	483 (401–654)	526 (428–676)	465 (377–621)	0.31
Median estimated days postinfection (IQR)	59 (36–72)	60 (40–72)	51 (32–96)	0.75

[a] Protective HLA alleles defined as B*13, B*27, B*57, and B*5801.
[b] *P* values determined by a Mann-Whitney U test.
[c] IQR, interquartile range.

AGGCG AGGGG CGGCG ACTGG TGAGT ACGCC AAAAA TTTTG ACTAG CGGAG GCTAG AAGGA GAGAG ATGGG) and downstream of protease (reverse primer GGCCC AATTT TTGAA ATTTT TCCTT CCTTT TCCAT TTCTG TACAA ATTTC TACTA ATGCT TTTAT TTTTT CTTCT GTCAA TGGCC ATTGT TTAAC TTTTG).

Plasmid pNL4-3ΔGag-Protease was developed by inserting unique BstEII restriction sites at the 5′ end of Gag and the 3′ end of the protease by using the QuikChange XL kit (Stratagene), followed by the deletion of the Gag-Protease region by BstEII digestion (New England Biolabs). This plasmid was maintained by using *Escherichia coli* Stbl3 cells (Invitrogen). To generate recombinant viruses, 10 μg of BstEII-linearized plasmid plus 50 μl of the second-round amplicon (approximately 5 μg) were mixed with 2.0×10^6 cells of a Tat-driven green fluorescent protein (GFP) reporter T-cell line (GXR 25 cells [41]) in 800 μl of $R10^+$ medium (RPMI 1640 medium containing 10% fetal calf serum [FCS], 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) and transfected by electroporation using a Bio-Rad GenePulser II instrument (exponential protocol of 300 V and 500 μF). Following transfection, cells were rested for 45 min at room temperature, transferred into 25-mm^3 flasks in 5 ml of $R10^+$ medium, and fed with 5 ml $R10^+$ medium on day 5. GFP expression was monitored by flow cytometry (FACSCalibur; BD Biosciences), and once GFP-positive (GFP^+) expression reached >15% among viable cells, supernatants containing the recombinant viruses were harvested, and aliquots were stored at −80°C.

Replication capacity assays. Virus titers and replication assays were performed as described previously (47, 49). Replication assays were initiated at a multiplicity of infection (MOI) of 0.003 and included six negative (uninfected cells only) and six NL4-3 infection controls. For each virus, the natural-log slope of the percentage of GFP^+ cells was calculated during the exponential phase of viral spread (days 3 to 6). This value was divided by the mean rate of spread of wild-type (WT) NL4-3 to generate a normalized, quantitative measure of the replication capacity (RC). An RC value of 1.0 indicates a rate of viral spread that was equal to that of NL4-3, while RC values of <1.0 and >1.0 indicate rates of spread that were higher than or lower than those of NL4-3, respectively. Duplicate or triplicate assays were performed in independent experiments, and average replication rates are reported. Quality control experiments were done to test assay reproducibility and to assess the potential impact of recombination on the NL4-3 RC. RC values determined using bulk products were highly concordant for a given patient sample. Furthermore, the reintroduction of cloned NL4-3 sequences into the NL4-3 backbone did not significantly alter viral fitness.

Viral sequencing and sequence analysis. Bulk plasma HIV-1 RNA sequences were previously collected for all individuals in the acute/early (11) and chronic (14) infection cohorts. Recombinant viral stocks for all acute/early infections and the majority (528 of 803; 65.7%) of chronic viruses were sequenced to confirm patient origin and to assess diversity. Bulk HIV-1 RNA was extracted from viral culture supernatants by using the QIAamp viral RNA kit (Qiagen), amplified by nested RT-PCR using sequence-specific primers, sequenced bidirectionally on an ABI 3730xl sequencer (Applied Biosystems), and analyzed by using Sequencher 4.9 software (Gene Codes). Nucleotide mixtures were called if the secondary-peak height exceeded 25% of the dominant-peak height. All viruses were confirmed as subtype B by comparison to reference sequences (http://www.hiv.lanl.gov). Nucleotide alignments were performed by using a modified NAP algorithm (32), and maximum likelihood phylogenetic trees were generated by using PHYmL (30). Trees were visualized by using Figtree v.1.2.2 (http://tree.bio.ed.ac.uk/software/figtree). Chronic HIV-1 sequences were previously deposited in GenBank (14, 47).

Statistical analysis. All statistical analyses are identified in the text. An unpaired *t* test was used to compare differences in replication capacity between groups (e.g., presence versus absence of HLA alleles). The relationship between the number of HLA-associated escape mutations and RC was assessed by using Pearson's correlation, while Spearman's correlation was used to investigate the relationship between HIV-1 clinical parameters (CD4 cell count and pVL) and RC. In an exploratory analysis, pairwise Mann-Whitney U tests were used to identify specific amino acids in Gag and protease associated with RC. Multiple tests were addressed by using a *q* value approach (60).

Nucleotide sequence accession numbers. Data for acute/early viral sequences have been deposited in the GenBank database under accession numbers GU390464 to GU390529.

RESULTS

We have utilized a high-throughput recombinant virus assay to examine the impact of HLA-associated mutations in the Gag and protease proteins on viral replication capacity (RC) in order to elucidate the consequences of immune-mediated pressure on HIV-1 fitness during the natural course of infection.

Construction of Gag-Protease recombinant viruses from acute/early and chronic infections. Recombinant viruses were generated from 66 acute/early-infected individuals and 803 untreated individuals at a relatively late stage of chronic infection (Tables 1 and 2). The Gag regions of all acute/early viruses and the majority of chronic viruses were resequenced to confirm patient origin and to assess viral diversity. As expected, limited diversity was observed in the acute/early virus bulk

TABLE 2. Chronic cohort information

Parameter	Value for chronic cohort			*P* value[b]
	Total	Protective HLA allele[a]	No protective HLA allele	
No. of individuals	803	165	638	
Median $\log_{10}$ plasma viral load (IQR[c])	5.08 (4.65–5.46)	4.96 (4.42–5.43)	5.11 (4.72–5.47)	0.02
Median CD4 cell count (IQR)	273 (130–420)	290 (120–430)	270 (130–420)	0.91

[a] Protective HLA alleles defined as B*13, B*27, B*57, and B*5801.
[b] *P* values determined by a Mann-Whitney U test.
[c] IQR, interquartile range.

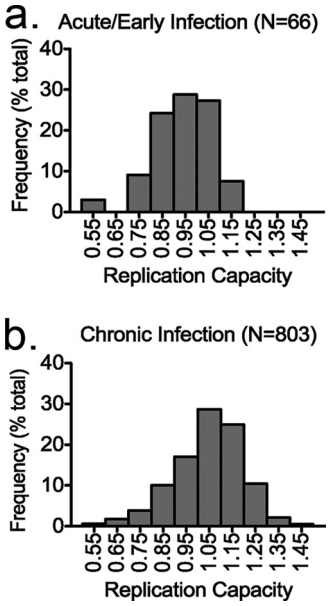

FIG. 1. *In vitro* RCs of recombinant NL4-3-derived viruses encoding patient-derived Gag-Protease sequences. The distribution of viral RC is shown for samples collected during acute/early infection (n = 66) (a) and chronic infection (n = 803) (b). Viruses were generated and RCs were measured by using a GFP reporter T-cell line, as described in Materials and Methods. The average RC was determined for each recombinant virus using data from two (acute/early infection) or three (chronic infection) independent assays. Results were normalized to mean RCs of WT NL4-3 controls assessed in parallel such that values greater than or less than 1.0 indicate viruses that replicated faster or slower than NL4-3, respectively. Gag-Protease RC values were not significantly different between cohorts (the median RC was 1.01 for both cohorts) and were distributed normally around means of 0.98 (±0.12) for acute/early infection samples and 1.00 (±0.15) for chronic infection samples.

plasma HIV-1 RNA as well as recombinant virus sequences (~55% of plasma and recombinant viruses were clonal at the amino acid level, while the remainder exhibited at least one amino acid mixture). However, a comparison of chronic plasma versus recombinant viral sequences revealed reduced viral diversity in the latter compared to that of the former: 6% of plasma versus 40% of recombinant sequences were clonal at the amino acid level ($P < 0.0001$), which is indicative of an *in vitro* genetic bottleneck, likely at the homologous recombination stage. Despite reduced diversity, recombinant viral sequences were highly concordant with the original bulk plasma sequences: the median number of full amino acid differences observed between plasma and recombinant virus was 1 out of 500 codons in Gag (<0.2% [IQR, 0% to 2%]).

In vitro RC was measured for each Gag-Protease recombinant virus as described previously (47, 49). The NL4-3 normalized median RC was highly concordant between cohorts (1.01 [IQR, 0.91 to 1.07] for acute/early viruses and 1.01 [IQR, 0.91 to 1.10] for chronic viruses; $P = 0.29$) (Fig. 1) and indicated that, on average, the *in vitro* function of patient-derived Gag-Protease sequences was comparable to that of NL4-3. To investigate the relationship between recombinant virus quasispecies diversity and RC, we compared the RC of acute/early viruses encoding clonal Gag sequences (n = 37) to those containing at least one amino acid mixture (n = 29), and we observed no significant difference in RCs between the two (P = 0.51). A similar analysis undertaken on the chronic virus data set revealed a slight growth advantage for viruses containing amino acid mixtures: the median RC of chronic recombinant viruses with clonal Gag sequences (n = 210) versus that for viruses with at least one amino acid mixture in Gag (n = 318) was 0.98 versus 1.01, respectively ($P = 0.01$). Taken together, these data suggest that recombinant stocks containing more than one viral species replicated marginally better in our cell culture system; however, the magnitude of this effect (0.03 units, equivalent to 20% of 1 standard deviation in the data set) was relatively minor.

Reduced RC of acute/early viruses from individuals expressing certain protective HLA alleles. Based on the results of previous studies (9, 10, 42, 46, 53, 59), we hypothesized that if viral escape mutations in Gag were to significantly reduce HIV-1 fitness, this would most likely occur early following infection and in the context of immune pressure elicited by protective HLA class I alleles. To assess this, we divided acute/early infection samples into two groups based upon host expression of protective HLA alleles that are known to target epitopes in Gag (B*13, B*27, B*57, and B*5801) (16, 31). The time of sample collection postinfection and the proportion of recombinant viruses exhibiting clonal sequences were similar between groups (Table 1 and not shown). However, pVL was significantly lower for acute/early-infected individuals who expressed a protective HLA allele than for those who did not (Table 1). Notably, we observed that recombinant Gag-Protease viruses derived from individuals who expressed at least one of these protective HLA alleles demonstrated significantly lower *in vitro* RCs than those who did not ($P < 0.0001$) (Fig. 2a). In an analysis stratified by the individual HLA-B alleles expressed, viruses derived from HLA-B*57, -B*5801, and -B*13 displayed the lowest RCs overall (Fig. 2b). Note that one individual expressed both B*5801 and B*13, but similar results were observed after the removal of this sample (not shown). Stratification by expressed HLA-A and HLA-C alleles revealed no significant associations with Gag-Protease RC (not shown).

Evidence for early immune selection as a correlate of reduced RC. We hypothesized that alterations in the RC in acute/early infection were due to the rapid selection of immune escape mutations by host HLA alleles. We assessed this hypothesis in three ways. First, we investigated the presence of HLA-associated escape mutations previously associated with reduced fitness in the four viruses with the lowest overall RC values in the cohort (which were derived from individuals expressing B*5701 [n = 2], B*1302, and B*5801) and compared these viruses to viruses derived from the remaining individuals who expressed B*57/B*5801 (n = 4) or B*13 (n = 4) (9, 10, 42, 53). All four viruses exhibiting the lowest RC values harbored mutations previously associated with reduced fitness, including mutations at Gag residues 147 (2 of 3 viruses from B*57- or B*5801-expressing individuals), 242 (3 of 3 viruses), 248 (2 of 3 viruses), and 340 (1 of 3 viruses). An HLA B*57/B*5801-associated Gag mutation at codon 173 was also seen for all three of these viruses; however, to our knowledge, the consequence of this mutation on fitness has not been evaluated. Furthermore, the recombinant virus derived from the B*13-

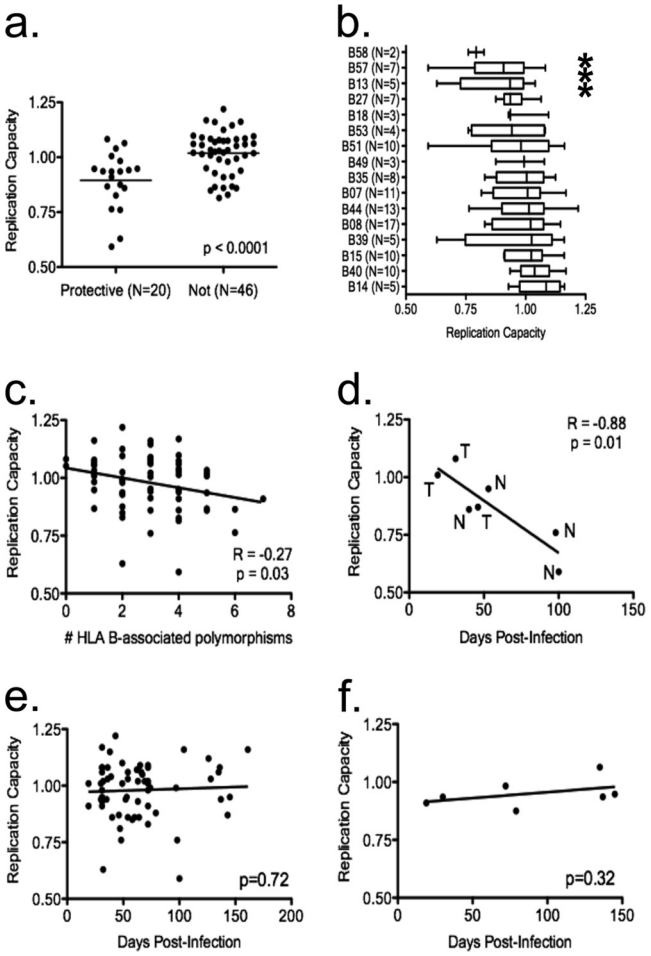

FIG. 2. Host HLA-B expression is associated with Gag-Protease RC in acute/early infection. (a) Significantly lower RCs were observed among viruses derived from individuals who expressed HLA-B*13, -B*27, -B*57, and/or -B*5801 ("protective") ($n = 20$) than among viruses derived from individuals who lacked these HLA alleles ("non-protective") ($n = 46$) ($P < 0.0001$ by a t test). The median RC for each group is indicated by a horizontal line. (b) Host expression of HLA-B*13, -B*57, and -B*5801 contributed to lower RCs ($P = 0.04$, $P = 0.01$, and $P = 0.02$, respectively, by a t test) (indicated by asterisks), while the expression of B*27 did not ($P = 0.50$). Results for individual alleles were not significant after correction for multiple comparisons (all $q > 0.2$). Median RC values (lines), interquartile ranges (boxes), and extreme values (whiskers) are indicated for each group. (c) Total number of Gag polymorphisms associated with host HLA-B alleles inversely correlated with the RC ($R = -0.27$; $P = 0.03$). (d) Among seven acute/early viruses derived from HLA-B*57-expressing hosts, a significant inverse correlation was observed between RC and the estimated time postinfection when the sample was collected ($R = -0.88$; $P = 0.01$). The viral sequence at residue 242 (T or N) is indicated as a reference. (e and f) No similar association with time was observed among viruses for the entire cohort (e) or among viruses derived from HLA-B*27-expressing hosts (f). The slope of each association is indicated by a solid line.

expressing individual harbored a known escape mutation at Gag codon 437 within the B*13-RI9 ($Gag_{428-437}$) epitope. In contrast, these polymorphisms were observed less frequently in the more-fit recombinant viruses derived from the remaining individuals expressing these alleles, including those at codon 147 (1 one 4 viruses), 173 (1 of 4 viruses), 242 (1 of 4 viruses), 248 (2 of 4 viruses), or 340 (0 of 4) in the presence of B*57/B*5801 and at codon 437 (0 of 4 viruses) in the presence of B*13. These data are consistent with the *de novo* selection of escape mutations by host HLA-restricted CTL responses in each of these cases.

Second, we used a previously reported list of HLA-associated polymorphisms derived from statistical analyses of an international cohort of more than 1,500 antiretroviral-naïve, chronically infected individuals, which incorporated a correction for HLA linkage disequilibrium and HIV codon covariation (14), to explore the relationship between HLA-associated Gag polymorphisms and viral RC in a more systematic way. We observed a significant inverse correlation between the total number of host HLA-associated polymorphisms in acute/early Gag sequences and RC ($R = -0.30$; $P = 0.01$) (not shown). This result appeared to be driven largely by polymorphisms selected in the presence of the host's HLA-B alleles ($R = -0.27$; $P = 0.03$) (Fig. 2c). Significant correlations between RC and HLA-A- or HLA-C-associated Gag polymorphisms were not observed (not shown).

Finally, we hypothesized that CTL-mediated selection of escape mutations will occur gradually and therefore that changes in viral RC would become more apparent over time following infection. We therefore correlated RC with the estimated time postinfection when each sample was collected. Despite the cross-sectional nature of this analysis and limited data, we observed a significant inverse correlation between RC and days postinfection for the seven viruses derived from HLA B*57-expressing individuals ($R = -0.88$; $P = 0.01$) (Fig. 2d). This result is consistent with an effect of B*57-mediated immune pressure on Gag-Protease that results in the selection of escape mutations that attenuate viral fitness during the first months following infection. Indeed, viruses derived from earlier samples (collected at 19, 31, and 46 days postinfection) encoded the wild-type T at position 242, while those derived from later samples (days 40, 53, 98, and 100) contained the T242N escape mutation within the B*57-TW10 ($Gag_{240-249}$) epitope. In contrast to B*57-expressing individuals, no association between RC and time since infection was observed when we examined the entire cohort (Fig. 2e) or when we assessed viruses derived from individuals expressing B*27 ($n = 7$) (Fig. 2f), suggesting that the early attenuation of HIV-1 may be limited to a relatively small subset of HLA alleles. Altogether, these data support a dominant influence of some host HLA-B alleles, particularly B*57, on Gag-Protease fitness in early infection.

No evidence for transmitted escape mutations as a correlate of reduced RC. The acquisition of HIV-1 harboring Gag polymorphisms restricted by protective HLA alleles has been shown to affect acute-phase viral load (17, 27), presumably through alterations in viral fitness. We therefore investigated the relationship between transmitted mutations in Gag-Protease and viral RC. Using the same list of HLA-associated polymorphisms described above, we identified mutations in each virus that were likely to be selected in the previous host. However, we observed no significant correlations between acute/early viral RC and the total number of Gag polymorphisms associated with these putative transmitted escape mutations (not shown).

Identification of specific mutations associated with acute/early virus RC. To identify specific amino acids in Gag-Protease associated with acute/early virus RC, we performed a systematic pairwise analysis of all observed amino acid variants and viral RCs. This analysis revealed correlations between RC and 18 polymorphisms located at 14 codons in Gag ($P < 0.05$), although none reached statistical significance after correction for multiple comparisons (all $q > 0.2$) (see Table S1 in the supplemental material). A lower median RC was observed for strains encoding the Gag V7I, S53T, S67A, E260D, S342X, Y484X, or L486X mutation as well as those encoding a deletion at residue T371. Notably, several consensus subtype B residues in Gag were also seen to be significantly associated with a lower RC, including L34, Q55, L75, V218, I479, and L498, which may help to explain the observation that many patient-derived viruses displayed higher RCs than did the NL4-3 control. Polymorphisms at a number of these sites were associated previously with HLA-mediated selection in Gag (14), and well-documented CTL epitopes overlap many of these regions (see the HIV molecular immunology database at www.hiv.lanl.gov). Despite its association with reduced RCs, no HLA B*57-associated polymorphisms were observed at a P value of <0.05, although trends were seen for Gag polymorphisms at several known B*57-targeted residues, including the T242N (present in 6 of 66 viruses; $P = 0.06$) and I147L (present in 16 of 66 viruses; $P = 0.11$) mutations.

Contribution of host HLA alleles to viral RC in chronic infection. In light of the observed effects of host HLA on the Gag-Protease RC during acute/early infection, we wished to investigate whether HLA-associated viral attenuation persisted into later stages of infection. To do this, we constructed and evaluated a large panel of recombinant viruses ($n = 803$) from untreated individuals with relatively advanced chronic infection (Table 2). In contrast to acute/early infection results, no significant association was observed between host expression of protective HLA alleles (B*13, B*27, B*57, and B*5801) and chronic viral RC ($P = 0.28$) (Fig. 3a) despite a greatly increased power to detect such differences. Furthermore, the stratification of data by individual HLA alleles also failed to reveal associations between most of these alleles and RC (Fig. 3c). Of interest, the stratification of the RC by individual HLA alleles revealed a relatively broad range of RC values for HLA-B (from medians of 0.89 for B*56 to 1.06 for B*37) (Fig. 3c) but narrower ranges for HLA-A and -C (Fig. 3b and d), supporting a greater influence of HLA-B on chronic viral fitness. HLA alleles A*26, A*31, B*48, B*53, and B*5801 were independently associated with lower viral RCs in chronic infection (all $q < 0.2$).

Impact of the number of HLA-associated polymorphisms on viral RC in chronic infection. In contrast to acute/early infection, no significant relationship was observed between the overall total number of HLA-A-, HLA-B-, or HLA-C-associated polymorphisms and viral RC in chronic infection (not shown). Due to the substantially increased power in the chronic cohort, it was additionally possible to undertake these analyses at the individual HLA allele level. Of interest, we observed significant inverse correlations between RC and the number of HLA-specific polymorphisms restricted by A*25 ($n = 30$) ($R = -0.37$; $P = 0.04$), A*26 ($n = 46$) ($R = -0.35$; $P = 0.02$), B*14 ($n = 50$) ($R = -0.30$; $P = 0.04$), B*41 ($n = 12$) ($R = -0.89$; $P < 0.0001$), and B*55 ($n = 33$) ($R = -0.53$; $P = 0.002$) for individuals expressing these alleles (Fig. 4). Associations for HLA-A*26, -B*41, and -B*55 remained significant after correction for multiple comparisons ($q < 0.2$). These results suggest a potential dose-dependent relationship between the induction of escape mutations by these HLA alleles and reduced RCs in some individuals. Notably, A*26 was also associated with a lower overall RC (Fig. 3c). The finding that these other alleles are not broadly associated with reduced fitness suggests that their effects may be driven by Gag responses that are poorly elicited in many cases or are due to atypical escape mutations that result in greater viral attenuation.

Since the transmission of HLA-associated Gag mutations may alter viral fitness, the potential impact of polymorphisms that could not be attributed to the current host's HLA profile (i.e., escape mutations acquired at transmission) was also ex-

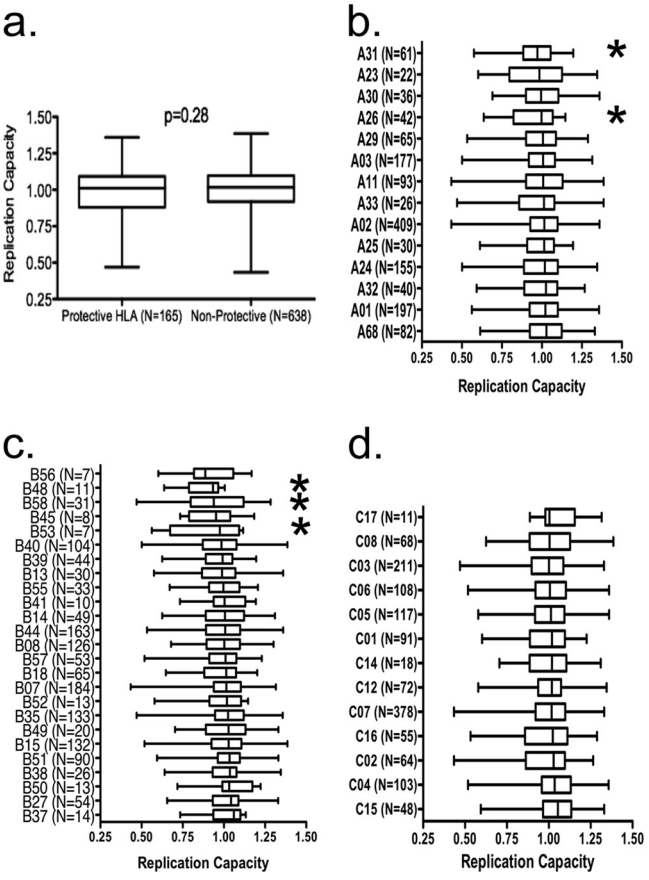

FIG. 3. Lack of strong associations between protective HLA alleles and RC in chronic infection. (a) The RC of viruses derived from chronically infected individuals who expressed the protective HLA alleles B*13, B*27, B*57, and/or B*5801 was not significantly different from those of viruses derived from individuals who did not express these alleles. (b and c) When host HLA alleles were analyzed individually, several alleles were associated with significantly lower Gag-Protease viral RCs after correction for multiple comparisons ($q < 0.2$) (indicated by asterisks), namely, A*26 and A*31 (b) as well as B*48, B*53, and B*58 (c). (d) No associations with chronic infection RC were observed for HLA-C alleles. Median RC values (lines), interquartile ranges (boxes), and extreme values (whiskers) are indicated for each group.

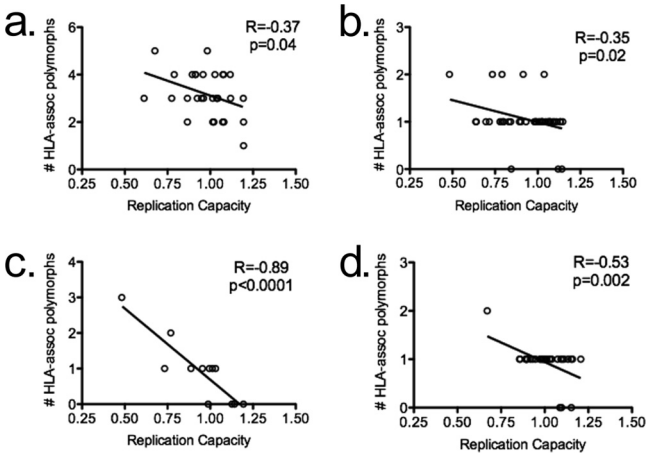

FIG. 4. Cumulative effects of HLA-associated polymorphisms on viral RC during chronic infection. RC measurements were compared to the total number of Gag mutations present in each virus that could be attributed to the host's HLA, as defined previously (14). Significant associations were observed between the number of HLA-associated polymorphisms specific for A*25 (Pearson $R = -0.37$; $P = 0.04$) (a), A*26 ($R = -0.35$; $P = 0.02$) (b), B*41 ($R = -0.89$; $P < 0.0001$) (c), and B*55 ($R = -0.53$; $P = 0.002$) (d) and lower RCs in individuals who expressed these alleles, suggesting a dose-dependent impact of new Gag polymorphisms selected for by these HLA alleles. Associations for HLA-A*26, -B*41, and -B*55 remained significant after correction for multiple comparisons ($q < 0.2$). The slope of each association is indicated by a solid line.

amined. Consistent with results from acute/early infection studies, no associations were observed between the viral RC and the total number of HLA-A-, HLA-B-, or HLA-C-associated polymorphisms for alleles that were not expressed by the host (not shown). This result suggests that putative transmitted mutations could be observed for chronic viruses but that these polymorphisms were not a major determinant of RC for this cohort. However, the possibility that fitness-reducing mutations were transmitted and subsequently reverted cannot be ruled out.

Associations between Gag sequence polymorphisms and viral RC in chronic infection. A systematic analysis of viral amino acids associated with the RC in chronic infection was also undertaken. A total of 63 polymorphisms located at 40 codons in Gag and two polymorphisms located at codon 61 in protease that were associated with viral RC at a q value of <0.2 were identified (see Table S2 in the supplemental material). Of the Gag codons associated with viral RC, 22 (55%) are under selection pressure by at least one HLA allele (14). Polymorphisms correlating with lower viral RCs in chronic infection included Gag E12Q (associated with B*49), K28T (A*03, A*24, and C*17), R30K (B*15), R58G (B*49), S67A (C*03), T122I (A*33), A146P (B*13, B*39, B*57, and C*08), S148T (B*53, C*02, C*06), V218Q (B*40), V223L (A*25, B*55, B*56), F383T (A*31 and B*27), T389I (B*13, B*27, B*39, B*42, and B*44), T427P (A*26, B*40, and B*58), and E482D (B*40 and B*78). Of note, changes at Gag codons 67, 218, 479, and 486 were identified as being associated with RC in both the acute/early and chronic infection data sets.

Impact of secondary mutations in Gag on the B*57-associated T242N mutation in chronic infection. In contrast to acute/early infection, B*57-derived recombinant viruses did not display significantly reduced RCs in chronic infection. In order to further investigate this observation, we examined chronic viral sequences for the presence of HLA B*57-associated polymorphisms. As expected, we observed a significant enrichment of known Gag CTL escape mutations, A146P, I147L, T242N, and others, in chronically infected B*57-expressing individuals; however, we failed to observe a significant correlation between the overall number of B*57-associated polymorphisms in these sequences and viral RC (not shown). Taken together, these observations indicate that during chronic infection, the relationship between viral sequence and RC is more complex than the raw number of primary escape mutations.

We therefore hypothesized that the lack of a B*57 association with RC at the chronic stage was due to the accumulation of compensatory mutations during the natural course of infection. To assess the potential relationship between compensatory mutations and viral RC in chronic infection, we examined the sequences of 50 viruses derived from B*57-expressing individuals harboring the T242N mutation for evidence of secondary mutations at Gag H219Q, I223V, M228I, and G248A. These mutations have been shown to restore the *in vitro* RC of T242N variants, presumably by altering the ability of capsid to interact with the host cyclophilin A protein (10, 41, 42). In viruses encoding T242N, we observed a significant positive correlation between RC and the number of compensatory mutations in the recombinant viral sequence ($R = 0.36$; $P = 0.01$) (Fig. 5a). Similar results were obtained if all T242N mutation-containing viruses (regardless of the host HLA type) were analyzed ($n = 72$; $R = 0.33$; $P = 0.004$) (not shown). Together, these data indicate that the effects of the T242N escape mutation on RC may be restored by secondary mutations in a cumulative manner.

Limited experimental data exist to show the consequence of secondary mutations following CTL escape on HIV-1 fitness. We hypothesized that other polymorphisms in Gag might alter the impact of the T242N mutation, and therefore, we examined our chronic data set for evidence of additional secondary mutations that were associated with either an enhanced or reduced RC in context of T242N. Polymorphisms at Gag codons 12 ($P = 0.027$), 127 ($P = 0.027$), 146 ($P = 0.003$), 315 ($P = 0.015$), 483 ($P = 0.015$), and 488 ($P = 0.016$) were associated with lower RCs, indicating a deleterious effect of these mutations in combination with T242N (Fig. 5b). Polymorphisms at residues 146 and 315 are consistent with escape from HLA B*57-restricted CTL responses to the IW9 ($Gag_{147\text{-}155}$) and QW9 ($Gag_{308\text{-}316}$) epitopes, but only Gag codon 146 remained significant after correction for multiple comparisons ($q < 0.2$). These results support previous work indicating an additive effect of B*57-mediated escape mutations on viral fitness (9, 19).

In addition, we observed that polymorphisms at Gag codons 84 ($P = 0.005$), 132 ($P = 0.008$), 218 ($P = 0.030$), 219 ($P = 0.015$), 228 ($P = 0.0006$), 248 ($P = 0.002$), 370 ($P = 0.031$), and 487 ($P = 0.014$) were associated with higher RCs in T242N mutation-containing viruses (Fig. 5c). Of these polymorphisms, those present at codons 219, 228, and 248 were described previously to function as compensatory mutations for the T242N mutation (10, 41, 42). Note that only codons 228 and 248 remained significant after correction for multiple compar-

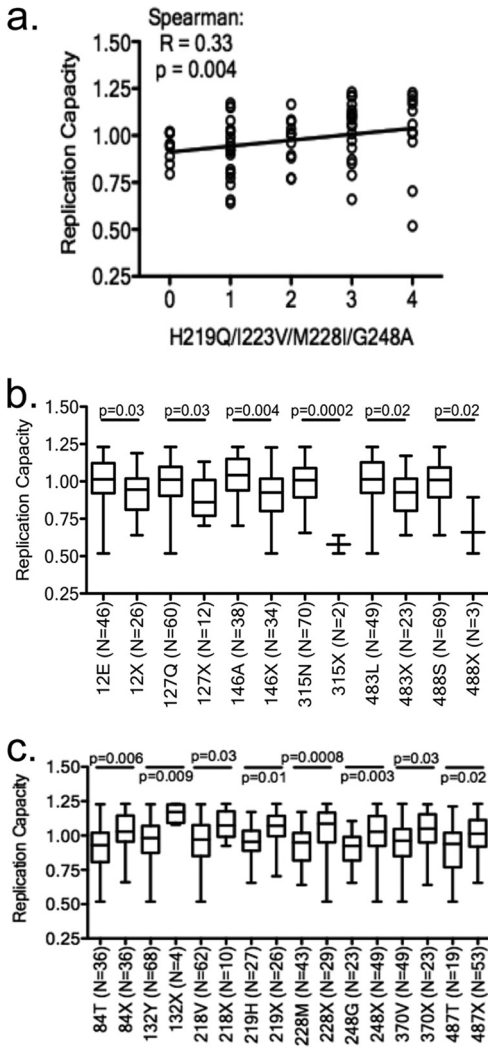

FIG. 5. Impact of secondary mutations in Gag on RC of chronic viruses carrying the T242N mutation. The presence of Gag H219Q, I223V, M228I, and G248A mutations was examined for recombinant viruses derived from B*57-expressing hosts that carried the T242N mutation ($n = 50$). (a) A significant correlation was observed between the RC and the total number of sites harboring previously described compensatory mutations (Pearson $R = 0.36$; $P = 0.01$). The slope of the association is indicated by a solid line. To identify novel secondary mutations that might alter the fitness of T242N mutation-carrying viruses, pairwise Mann-Whitney U tests were conducted to compare the RCs of 72 chronic viruses from all patients that harbored the T242N mutation with all variable residues in Gag. (b and c) Results indicated significant associations between mutations at Gag codons 12, 127, 146, 315, 483, and 488 with lower RCs (b) and between mutations at Gag codons 84, 132, 218, 219, 228, 248, 370, and 487 with higher RCs (c) (all $P < 0.05$). Associations at codons 146, 228, and 248 remained significant after correction for multiple comparisons ($q < 0.2$). Median RC values (lines), interquartile ranges (boxes), and extreme values (whiskers) are indicated for each group.

isons ($q < 0.2$). Altogether, our results confirm and extend the list of secondary mutations in Gag that might enhance or compensate for fitness costs associated with the T242N mutation.

Clinical associations with viral RC in chronic infection. Finally, we wished to examine associations between Gag-Protease RC and clinical parameters (Fig. 6). A modest positive correlation was observed between RC and plasma viral load (pVL) ($R = 0.12$; $P = 0.0007$), while an inverse correlation was observed between RC and the CD4 cell count ($R = -0.17$; $P < 0.0001$) in chronic infection. Restricting this analysis to individuals expressing protective HLA alleles associated with lower acute/early fitness in our study (B*13, B*57, or B*5801) ($n = 110$) revealed stronger associations between the RC and pVL ($R = 0.27$; $P = 0.005$) and CD4 cell counts ($R = -0.33$; $P = 0.0005$). Although these analyses are derived from cross-sectional data, results suggest that Gag fitness may increase over the course of chronic infection. The stronger Rho obtained from the analysis of protective alleles suggests that the increase in viral RC over the infection course may be greater for these alleles than for others, which is consistent with our observation of reduced RCs in acute/early infection with recombinant viruses derived from individuals expressing protective alleles.

DISCUSSION

An effective AIDS vaccine must overcome the extreme genetic diversity of HIV-1 (4, 26, 61). Targeting of the most conserved regions of the viral proteome has been proposed as a means to elicit robust, long-lasting CD8 T-cell responses. Indeed, highly conserved HIV-1 epitopes that escape very slowly during natural infection have been identified, for example, the B*57-restricted KF11 ($Gag_{162-172}$) (20) and B*27-restricted KK10 ($Gag_{263-272}$) (25, 29) epitopes. Structural constraints on these regions of the viral capsid likely require compensatory mutations to occur concurrently in order for viruses encoding these altered epitopes to remain viable (20, 58, 59, 65). A vaccine approach that targets these and other regions of Gag may also force HIV-1 to adopt an attenuated phenotype through the selection of detrimental CTL escape mutations. This seems plausible, since some conserved epitopes escape relatively rapidly in the presence of CTL pressure but revert to the wild-type sequence upon transmission to an HLA-mismatched recipient. The B*57-restricted TW10 ($Gag_{240-249}$) epitope is an example of one such case where escape mutations reduced viral fitness; however, this mutation also appears to be restored through the subsequent acquisition of compensatory mutations (8, 10, 42). The functional consequences of escape and potential pathways for compensation have been described for only a limited number of selected HIV-1 mutations, and data remain biased toward certain protective HLA alleles. Therefore, it is not known whether escape mutations selected in the presence of other alleles will display similar functional limitations that might be exploited for vaccine design.

Here, we have examined clinical Gag and protease sequences from individuals during acute/early infection and relatively advanced chronic infection to investigate the impact of HLA-associated immune selection pressures on HIV-1 fitness. We observed that Gag-Protease function, as indicated by the *in vitro* RC, was significantly lower for acute/early viruses derived from individuals who expressed a protective HLA class I allele, either B*13, B*57, or B*5801. This was associated with the presence of known HLA-associated polymorphisms in Gag that were expected in the context of each host's HLA class I

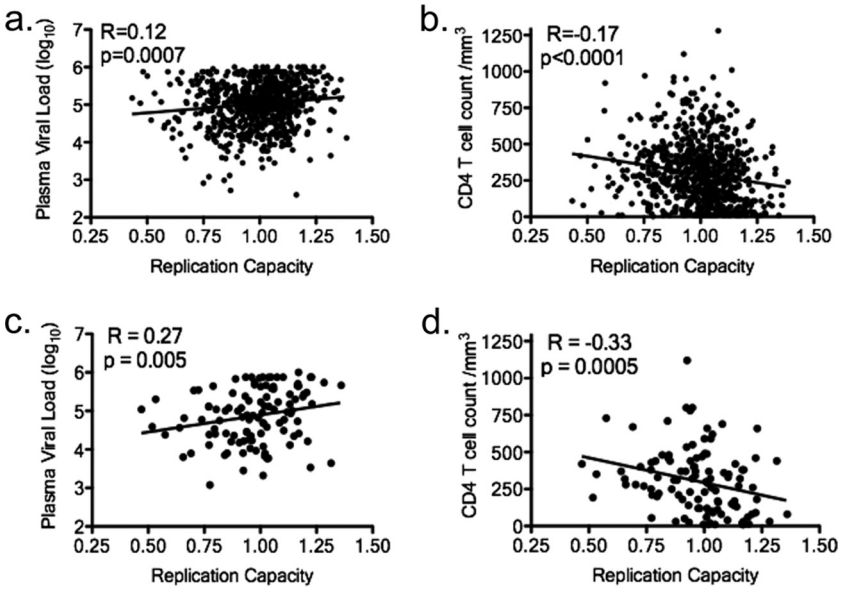

FIG. 6. Clinical associations with Gag-Protease RC in chronic infection. RC was compared to clinical pVL and peripheral blood CD4 T-cell counts at the time of collection for all chronic samples (a and b) and for the subset of samples collected from individuals who expressed a protective HLA allele, either B*13, B*57, or B*5801 ($n = 110$) (c and d). (a and b) Among all subjects, modest statistically significant correlations were observed between RC and pVL (a) (Pearson $R = 0.14$; $P < 0.001$) and CD4 cell count (b) ($R = -0.21$; $P < 0.0001$). (c and d) For individuals who expressed protective HLA alleles, we observed stronger associations between RC and pVL (c) ($R = 0.27$; $P = 0.005$) as well as CD4 cell count (d) ($R = -0.33$; $P = 0.0005$). The slope of each association is indicated by a solid line.

genotype. Notably, for viruses derived from acute/early-infected, B*57-expressing individuals, we observed that the RC correlated inversely with the number of days elapsed since the estimated infection date. Although based on a cross-sectional analysis, this result is consistent with the appearance of escape mutations in Gag early following infection (2, 7, 11, 21, 28, 44, 54) and their continued selection over time (29), leading to cumulative reductions in the viral replication capacity during the early stages of HIV-1 infection (8, 19). Our observation that the RC of viruses from acute/early infection correlated inversely with the total number of host HLA-B-associated polymorphisms present in Gag also supports this model. Taken together, these results suggest that an accumulation of primary escape mutations, selected predominantly by HLA-B alleles, contributes to reduced Gag function in a cumulative manner during acute/early infection. More detailed longitudinal analyses of Gag protein function will be necessary to evaluate this association further.

In an exploratory analysis of acute/early viruses, we identified polymorphisms at 14 codons that were significantly associated with viral RC (see Table S1 in the supplemental material). A majority of these sites (8 of 14 sites) were associated previously with HLA pressure (14), and all of them lay within known CTL epitopes. However, we were surprised to observe an overall lack of significant associations between RC and specific escape mutations restricted by protective HLA alleles. Trends were observed for several epitopes targeted by HLA-B*57/*5801, suggesting that the effect of these HLA alleles on RC may require multiple mutations to act in concert. The significant inverse association identified between the RC and the total number of host-specific HLA-B polymorphisms in Gag further suggests that the observed functional defects result from an additive effect of mutations, perhaps selected by more than one HLA-B allele during acute/early infection. It should be noted that our data set of 66 acute/early viruses was insufficiently powered to comprehensively identify specific amino acids associated with viral RC and to fully investigate potential correlations between RC, HLA, and clinical parameters.

Using a panel of 803 recombinant viruses generated from patients with chronic infection, we failed to observe an association between these same protective HLA alleles and Gag-Protease RC. However, a number of significant associations between RC and host HLA alleles were apparent. Our results indicated that host expression of HLA-A*26, -A*31, -B*48, -B*53, and -B*5801 may be associated with lower Gag function during chronic HIV-1 infection. Of these alleles, A*26, A*31, and B*5801 have been associated with a lower relative hazard for progression to AIDS in natural-history studies (51). In this study, only the host expression of HLA B*5801 correlated with a lower Gag-Protease RC in both acute/early infection and chronic infection.

The lack of a major association between HLA-B*57 and viral fitness in our chronic cohort may be due to the relatively late-stage infection of these individuals. In a previous study, we observed a modest association between HLA-B*57/B*5801 and a reduced Gag-Protease RC in a smaller cohort of chronically infected individuals with higher CD4 cell counts (48). Indeed, it is possible that the functional impact of early immune pressure by protective HLA class I alleles on Gag wanes over the disease course. This outcome is consistent with the ability of HIV-1 to evolve and to develop compensatory mutations, which appeared to be particularly relevant for the chronic infection samples analyzed here. Notably, we observed that nearly all viruses derived from chronically infected B*57-

and B*5801-expressing hosts harbored the Gag T242N escape mutation in the TW10 epitope and that the RCs of these strains correlated significantly with the accumulation of known and putative compensatory mutations in the capsid protein. Together with previously reported data from mutagenesis studies (10), these findings indicate that the accumulation of secondary mutations restores the fitness defects associated with primary escape mutations in a dose-dependent manner.

Despite a complex mixture of CTL escape and compensatory mutations present in chronic viral sequences, we observed a modest negative correlation between Gag-Protease function and CD4 T-cell count, which was strengthened upon restriction to protective HLA alleles only. Although these analyses are derived from cross-sectional data, results suggest that viral RC increases concomitantly as CD4 cell counts decline over the course of chronic infection. Indeed, the stronger correlations observed when analyses were restricted to protective HLA alleles suggest that changes in RC are greater over the course of chronic disease in the context of these alleles. This interpretation is consistent with our observation of a reduced RC in acute/early infection of individuals expressing protective HLA alleles, which is followed by an accumulation of compensatory changes that rescue the fitness costs of primary escape mutations. Unfortunately, we cannot fully explore this hypothesis, since infection dates are unknown for the chronic infection cohort. Efforts to examine RCs using longitudinal samples from well-characterized seroconverter cohorts will be necessary to address these issues further. Importantly, however, the fact that individuals with protective alleles exhibit reduced viral loads in chronic infection compared to those of their non-protective-allele-expressing counterparts strongly suggests that these early fitness hits, although no longer directly detectable at later time points due to the accumulation of compensatory mutations, may provide long-lasting beneficial effects.

The larger data set of recombinant viruses from chronically infected patients provided greater power to uncover specific amino acids in Gag and protease associated with RC. We identified 63 polymorphisms located at 40 codons in Gag and two polymorphisms located at codon 61 in protease that were independently associated with RC after correction for multiple comparisons ($q < 0.2$) (see Table S2 in the supplemental material). As seen for acute/early infection samples, the majority of these sites (22 of 42 sites) were previously associated with HLA-mediated selection (14), and many others lie within known CTL epitopes. However, few of these mutations matched the predominant HLA-associated polymorphisms identified in the published literature, possibly suggesting that less-common mutations at these sites are more likely to result in severe RC defects. Of interest, the strongest HLA associations with lower RCs in chronic infection were observed for HLA-A*31 and -B*40, and polymorphisms targeted by these alleles were also identified, namely, F383T (A*31) and E482D (B*40). Future studies will be necessary to examine the impact of these specific mutations on Gag RC and to assess their potential relevance for disease progression.

Polymorphisms at Gag codons 67, 218, 479, and 486 were associated with RC in both acute/early and chronic viruses. Of these polymorphisms, the S67A, V218X (acute/early) or V218Q (chronic), and L486X (acute/early) or L486S (chronic) mutations appeared to attenuate Gag RC, but interpretation of these results proved complex. For example, Gag position 67 appears to be under opposing selection pressures by different HLA alleles at the population level: while HLA-C*03 selects for S67A, HLA-A*02 selects for the wild-type serine at this residue (14). Mutations at codon 218 are associated with HLA-B*40, but this allele typically selects for alanine (rather than glutamine) at this residue (14). To our knowledge, mutations at codon 486 have not been identified as HLA footprints, although this residue lies within the known B*40-restricted KL9 epitope. Future site-directed mutagenesis studies will confirm these and other associations with viral RC so that they may be considered potential regions for inclusion or exclusion from Gag vaccine antigens designed to attenuate viral RC.

Several limitations of this study should be noted. First, we focused this work only on the Gag-Protease region of HIV-1. While we believe that the approach used provides a robust analysis of the impact of mutations in Gag and protease on the viral RC, we have not assessed important potential roles of polymorphisms located elsewhere in the viral genome. Second, due to the very large number of samples tested, we used bulk PCR products to construct recombinant viruses. The presence of amino acid mixtures in resulting stocks may have reduced our ability to detect minor differences in RC. Third, our acute/early infection study was limited to 66 individuals. This number was sufficient to observe a strong effect of protective HLA alleles on acute/early RC, but substantially larger acute/early infection cohorts may yield the ability to investigate RC associations with individual alleles and/or viral polymorphisms in greater detail.

In conclusion, this study and our previous report (64) focusing on subtype C infection represent the first systematic, population-based investigations of the contribution of HLA class I selection pressure on HIV-1 RC. Taken together, our findings are consistent with a model whereby HLA-associated CTL responses select for primary escape mutations in Gag during acute/early infection, some of which occur at a substantial fitness cost. Negative consequences for RC may be cumulative as additional escape mutations are selected; however, compensatory mutations that restore Gag function may also arise over extended periods of time. By late chronic infection, due to a balance of escape and compensatory mutations, many HLA-associated fitness defects observed during early infection are no longer detectable. A significant positive correlation between RC and the presence of compensatory mutations in chronic viruses illustrates the profound ability of continued *in vivo* viral evolution to rescue fitness defects. Nevertheless, the fact that individuals with protective alleles maintain lower pVLs than their non-protective-allele-expressing counterparts well into chronic infection strongly suggests that these early fitness hits may provide long-lasting benefits. These data provide important new information to better understand the complex interactions between HLA-mediated immune pressure and HIV-1 sequence evolution, the impact of escape and compensatory mutations on HIV-1 RC, and the potential utility of targeting attenuation-inducing sites in Gag for the rational design of an effective vaccine.

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M.A.B., Z.L.B., T.M., B.D.W., and T.M.A. designed this study. M.A.B, Z.L.B., J.S., P.C.R, and T.J.M. conducted experiments and analyzed data. C.J.B., C.M.K., J.M.C., and D.H. developed statistical methods and/or contributed to data analysis. H.S., A.D.K., H.J., E.R., M.M., M.A., and P.R.H. contributed specimens and/or clinical data. The manuscript was written by M.A.B. and Z.L.B. and critically reviewed by C.J.B., T.M., T.J.M., H.S., A.D.K., M.M., H.J., E.R., M.A., P.R.H., B.D.W., and T.M.A.

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